

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

DICOSIMO ET AL.

CASE NO.: CL1809 US NA

APPLICATION NO.: UNKNOWN

GROUP ART UNIT: UNKNOWN

FILED: CONCURRENTLY HEREWITH

EXAMINER: UNKNOWN

FOR: PRODUCTION OF CYCLIC TERPENOIDS

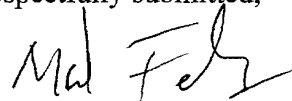
Assistant Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION IN ACCORDANCE WITH 37 CFR 1.821

I hereby state that the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 CFR 1.821(c) and (e), respectively are the same.

Respectfully submitted,



S. Neil Feltham
Attorney for Applicant
Registration No. 36,506
Telephone: 302-992-6460

Dated: 8/24/01

09938956 082401

TITLE

PRODUCTION OF CYLIC TERPENOID

This application claims the benefit of U.S. Provisional Application No. 60/229,907, filed September 1, 2000 and the benefit of U.S.

5 Provisional Application No. 60/229,858 filed September 1, 2000.

FIELD OF THE INVENTION

This invention is in the field of microbiology. More specifically, this invention pertains to methods for the production of cyclic terpenoid compounds in microbial hosts that metabolize single carbon substrates as
10 a sole carbon source.

BACKGROUND OF THE INVENTION

Monoterpenes have value in the flavor and fragrance industries, as components in industrial solvents and in the pharmaceutical industry where selected compounds have shown promise as both chemopreventive
15 and chemotherapeutic agents for solid tumors.

Although found in a wide range of organisms, including bacteria, fungi, algae, insects and even higher animals such as alligators and beavers, monoterpenes are most widely produced by terrestrial plants such as components of flower scents, essential oils, and turpentine. One
20 of the most common sources of the monoterpenes are the herbaceous plant and conifer turpentines. The pinene regioisomers (α -pinene, β -pinene) are 2 principal monoterpenes of turpentine as they serve as large volume aroma chemicals. Other essential oils (from orange, lime, lemon, and peppermint) are valued in flavoring and perfumery. The
25 cyclization of linear terpenoid compounds to form cyclic derivatives may generate diverse aromatic structures with differing functionality.

At present the monoterpenes may be obtained either by extraction from natural sources or by chemical synthesis. Both processes are time consuming and expensive. Although small scale production of selected
30 monoterpenes has been demonstrated in microbial hosts, a facile method for the production of monoterpenes on an industrial scale has yet to be reported. For example some monoterpene synthases have been successfully cloned and expressed in *Escherichia coli*. Specifically, limonene synthase, which catalyzes the cyclization of geranyldiphosphate
35 to yield the olefin 4(S)-limonene in *Perilla frutescens* has been cloned into *Escherichia coli* and functionally expressed (Yuba *et al. Arch Biochem Biophys* 332:280-287, (1996)). Reports of microbial expression however

have been limited to microbe traditionally used for fermentative production were grown on complex carbon substrates.

There are a number of microorganisms that utilize single carbon substrates as sole energy sources. These organisms are referred to as methylotrophs and herein as "C1 metabolizers". These organisms are characterized by the ability to use carbon substrates lacking carbon to carbon bonds as a sole source of energy and biomass. A subset of methylotrophs are the methanotrophs which have the unique ability to utilize methane as a sole energy source. Although a large number of these organisms are known, few of these microbes have been successfully harnessed to industrial processes for the synthesis of materials. Although single carbon substrates are cost effective energy sources, difficulty in genetic manipulation of these microorganisms as well as a dearth of information about their genetic machinery has limited their use primarily to the synthesis of native products. For example the commercial applications of biotransformation of methane have historically fallen broadly into three categories: 1) Production of single cell protein, (Sharpe D. H. *BioProtein Manufacture* 1989. Ellis Horwood series in applied science and industrial technology. New York: Halstead Press.) (Villadsen, John, *Recent Trends Chem. React. Eng.*, [Proc. Int. Chem. React. Eng. Conf.], 2nd (1987), Volume 2, 320-33. Editor(s): Kulkarni, B. D.; Mashelkar, R. A.; Sharma, M. M. Publisher: Wiley East., New Delhi, India; Naguib, M., Proc. OAPEC Symp. Petroprotein, [Pap.] (1980), Meeting Date 1979, 253-77 Publisher: Organ. Arab Pet. Exporting Countries, Kuwait, Kuwait.); 2) epoxidation of alkenes for production of chemicals (US 4348476); and 3) biodegradation of chlorinated pollutants (Tsien et al., *Gas, Oil, Coal, Environ. Biotechnol.* 2, [Pap. Int. IGT Symp. *Gas, Oil, Coal, Environ. Biotechnol.*], 2nd (1990), 83-104. Editor(s): Akin, Cavit; Smith, Jared. Publisher: Inst. Gas Technol., Chicago, IL.; WO 9633821; Merkley et al., *Biorem. Recalcitrant Org.*, [Pap. Int. In Situ On-Site Bioreclam. Symp.], 3rd (1995), 165-74. Editor(s): Hinchee, Robert E; Anderson, Daniel B.; Hoeppel, Ronald E. Publisher: Battelle Press, Columbus, OH; Meyer et al., *Microb. Releases* (1993), 2(1), 11-22). Even here, the commercial success of the methane biotransformation has been limited to epoxidation of alkenes due to low product yields, toxicity of products and the large amount of cell mass required to generate product associated with the process.

One of the most common classes of single carbon metabolizers are the methanotrophs. Methanotrophic bacteria are defined by their ability to use methane as a sole source of carbon and energy. Methane monooxygenase is the enzyme required for the primary step in methane activation and the product of this reaction is methanol (Murrell et al., *Arch. Microbiol.* (2000), 173(5-6), 325-332). This reaction occurs at ambient temperature and pressures whereas chemical transformation of methane to methanol requires temperatures of hundreds of degrees and high pressure (Grigoryan, E. A., *Kinet. Catal.* (1999), 40(3), 350-363; WO 2000007718; US 5,750,821). It is this ability to transform methane under ambient conditions along with the abundance of methane that makes the biotransformation of methane a potentially unique and valuable process.

Many methanotrophs contain an inherent isoprenoid pathway which enables these organisms to synthesize other non-endogenous isoprenoid compounds. Since methanotrophs can use one carbon substrate (methane or methanol) as an energy source, it is possible to produce monoterpenes at low cost. Furthermore, during the fermentation, volatile compounds can be easily removed as methane is passed through fermentation media. It is also advantageous to produce via bio-route since many of monoterpenes have chirality and it is difficult to control the synthesis and purification of specific chirally active compound in chemical synthesis.

A need exists therefore for a method of production of highly valuable monoterpenes from an inexpensive feedstock. Applicants have solved the stated problem by providing a C1 metabolizing microorganism having transformed with a gene encoding a cyclic terpene synthase, having the ability to produce to a variety of monoterpenes.

SUMMARY OF THE INVENTION

The invention provides a method for the production of a monoterpene comprising:

- a) providing a transformed C1 metabolizing host cell comprising:
 - (i) suitable levels of geranyl pyrophosphate; and
 - (ii) at least one isolated nucleic acid molecule encoding a cyclic terpene synthase under the control of suitable regulatory sequences;

(b) contacting the host cell of step (a) under suitable growth conditions with an effective amount of a C1 carbon substrate whereby a monoterpene compound is produced.

Preferred single carbon substrates of the present invention include
5 but are not limited to methane, methanol, formaldehyde, formic acid, methylated amines, methylated thiols, and carbon dioxide.

Preferred C1 metabolizers or facultative methylotrophs where obligate methanotrophic bacteria are most preferred. Most preferred C1 metabolizers are those obligate methanotrophs comprising a functional
10 Embden-Meyerof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme.

Preferred cyclic terpene synthases of the invention include but are not limited to limonene synthase, pinene synthase, bornyl synthase, phellandrene synthase, cineole synthase, and sabinene synthase.

15 In an alternate embodiment the invention provides for the expression of upper pathway isoprenoid genes for the downstream production of monoterpenes, the upper pathway isoprenoid genes selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (DXS); D-1-deoxyxylulose-5-phosphate reductoisomerase
20 (DXR); 2C-methyl-d-erythritol cytidyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (IspA) and Geranyltranstransferase (PyrG).

BRIEF DESCRIPTION OF THE DRAWINGS.

25 SEQUENCE DESCRIPTIONS, AND BIOLOGICAL DEPOSITS

Figure 1 shows the map of pTJS75:dxs:dxr:Tn5Kn plasmid containing truncated limonene synthase gene.

Figure 2 shows the gas chromatography analysis of limonene produced in *Methylomonas 16a* culture.

30 Figure 3 shows the examples of monoterpenes derived from geranyl diphosphate.

Figure 4 illustrates the upper isoprenoid pathway

Figure 5 shows the growth of *Methylomonas 16a* compared to the growth of *Methylococcus capsulatus* under identical growth conditions.

35 Figure 6 is a Schematic of Entner-Doudoroff and Embden-Meyerhoff pathways in *Methylomonas 16a* showing microarray expression results numerically ranked in order of decreasing expression level.

The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions, which form a part of this application.

The following sequences comply with 37 C.F.R. 1.821-1.825

5 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the
10 Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NO:1-4 are primer sequences.

15 SEQ ID NO:5 is the nucleotide sequence of plasmid pTJS75:dxS:dxR:Tn5Kn.

SEQ ID NO:6 is the nucleotide sequence of limonene synthase gene from *Mentha spicata* with 57 amino acid sequences deleted from N-terminal.

20 SEQ ID NO:7 is deduced amino acid sequence of limonene synthase gene used in SEQ ID NO:6.

Applicants made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure:

Depositor Identification Reference	International Depository Designation	Date of Deposit
<i>Methylobacter</i> 16a	ATCC PTA 2402	August 21 2000

25

DETAILED DESCRIPTION OF THE INVENTION

30 The present invention describes a method for the synthesis of monoterpenes in a recombinant methylotrophic or methanotrophic host. Monoterpenes are used in flavors and fragrances, coatings and nutrition and health applications.

The following definitions may be used for interpretation of the claims and the specification.

"Open reading frame" is abbreviated ORF.

"Polymerase chain reaction" is abbreviated PCR.

The term "isoprenoid" or "terpenoid" refers to the compounds or any molecule derived from the isoprenoid pathway including 10 carbon terpenoids (monoterpene) and their derivatives, such as limonene, pinene, sabinene, β -phellandrene, borneol, carotenoids and xanthophylls.

5 The term "isoprene subunit" refers to a basic 5 carbon unit of isopentenyl diphosphate that further condenses to form a terpenoid.

The term "cyclic terpene synthase" refers to an enzyme capable of using geranyl pyrophosphate as a substrate to produce a cyclic terpenoid compound.

10 The term "monoterpene" refers to any 10 carbon compound derived from geranyl pyrophosphate or its derivatives built upon 2 isoprene subunits (see Figure 3 for example).

The term "cyclic monoterpene" refers to a cyclic terpenoid derived from geranyl pyrophosphate having 10 carbon atoms.

15 The term "limonene synthase" refers to enzyme that catalyzes the conversion of geranyl pyrophosphate to (-)-Limonene.

The term "pinene synthase" refers to the enzyme that catalyzes the conversion of geranyl pyrophosphate to pinene.

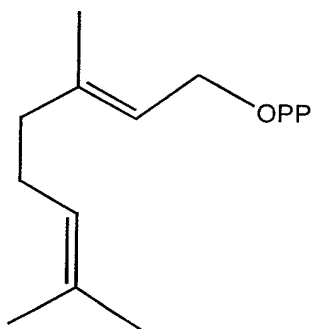
20 The term "bornyl synthase" refers to the enzyme that catalyzes the conversion of geranyl pyrophosphate to borneol.

The term "phellandrene synthase" refers to the enzyme that catalyzes the conversion of geranyl pyrophosphate to β -phellandrene.

The term "cineole synthase" refers to the enzyme that catalyzes the conversion of geranyl pyrophosphate to cineole.

25 The term "sabinene synthase" refers to the enzyme that catalyzes the conversion of geranyl pyrophosphate to sabinene.

The term "geranyl diphosphate" and "geranyl pyrophosphate" will be used interchangeably and will refer to a compound having the general formula



The term "single carbon substrate" refers to a carbon substrate useful as a microbial feedstock being devoid of carbon to carbon bonds.

The term "C1 metabolizer" refers to a microorganism that has the ability to use an single carbon substrate as a sole source of energy and biomass. C1 metabolizers will typically be methylotrophs and/or

The term "methylotroph" means an organism capable of oxidizing organic compounds which do not contain carbon-carbon bonds. Where the methylotroph is able to oxidize CH₄, the methylotroph is also a methanotroph.

The term "methanotroph" means a prokaryote capable of utilizing methane as a substrate. Complete oxidation of methane to carbon dioxide occurs by aerobic degradation pathways. Typical examples of methanotrophs useful in the present invention include but are not limited to the genera *Methylomonas*, *Methylobacter* *Methylococcus*, and *Methylosinus*.

The term "*Methylomonas 16a*" and "*Methylomonas 16a sp.*" are used interchangeably and refer to the *Methylomonas* strain used in the present invention.

The term "Embden-Meyerhof pathway" refers to the series of biochemical reactions for conversion of hexoses such as glucose and fructose to important cellular 3 carbon intermediates such as glyceraldehyde 3 phosphate, dihydroxyacetone phosphate, phosphoenol pyruvate and pyruvate. These reactions typically proceed with net yield of biochemically useful energy in the form of ATP. The key enzymes unique to the Embden-Meyerof pathway are the phosphofructokinase and fructose 1,6 bisphosphate aldolase.

The term "Entner-Doudoroff pathway" refers to a series of biochemical reactions for conversion of hexoses suchas as glucose or fructose to important 3 carbon cellular intermediates pyruvate and glyceraldehyde 3 phosphate without any net production of biochemically useful energy. The key enzymes unique to the Entner-Doudoroff pathway are the 6 phosphogluconate dehydratase and the ketodeoxyphosphogluconate aldolase.

The term "high growth methanotrophic bacterial strain" refers to a bacterium capable of growth with methane or methanol as sole carbon and energy source which possess a functional Embden-Meyerof carbon flux

pathway resulting in a high rate of growth and yield of cell mass per gram of C1 substrate metabolized. The specific "high growth methanotrophic bacterial strain" described herein is referred to as "*Methylomonas* 16a" or "16a", which terms are used interchangeably.

5 As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

10 "Gene" refers to a nucleic acid fragment that is capable of being expressed as a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is
15 not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found
20 in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A
25 "transgene" is a gene that has been introduced into the genome by a transformation procedure.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within,
30 or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector
35 binding site and stem-loop structure.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding

sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide.

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence

for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bannan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory Cold Press Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

The present invention provides a method for the synthesis of monoterpenes in a methylotrophic or methanotrophic microbial host. Typical monoterpenes of the invention are those that are derived from geranyl pyrophosphate and contain ten carbon atoms. Typically the hosts have the elements of the isoprenoid pathway that will result in the production of geranyl pyrophosphate. The microbial host will also comprise a gene encoding a synthase, which is capable of using geranyl pyrophosphate as a substrate to produce a monoterpene.

Identification and Isolation of C1 Metabolizing Microorganisms

The present invention provides for the expression of cyclic terpene synthases in microorganisms which are able to use single carbon substrates as a sole energy source. Such microorganisms are referred to herein as C1 metabolizers. The host microorganism may be any C1 metabolizer which has the ability to synthesize geranyl diphosphate (GPP), the precursor for many of the monoterpenes.

Many C1 metabolizing microorganisms are known in the art and are able to use a variety of single carbon substrates. Single carbon substrates useful in the present invention include but are not limited to methane, methanol, formaldehyde, formic acid, methylated amines (e.g. mono, di- and tri-methyle amine), methylated thiols, and carbon dioxide.

All C1 metabolizing microorganisms are generally classed as methylotrophs. Methylotrophs may be defined as any organism capable of oxidizing organic compounds which do not contain carbon-carbon bonds. A subset of methylotrophs are the methanotrophs which have the distinctive ability to oxidize methane. Facultative methylotrophs have the ability to oxidize organic compounds which do not contain carbon-carbon bonds, but may also use other carbon substrates such as sugars and complex carbohydrates for energy and biomass. Obligate methylotrophs are those organisms which are limited to the use of organic compounds which do not contain carbon-carbon bonds for the generation of energy and obligate methanotrophs are those obligate methylotrophs that have the ability to oxidize methane.

Facultative methylotrophic bacteria are found in many environments, but are isolated most commonly from soil, landfill and waste treatment sites. Many facultative methylotrophs are members of the β , and γ subgroups of the Proteobacteria (Hanson et al., *Microb. Growth C1 Compounds*, [Int. Symp.], 7th (1993), 285-302. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK; Madigan et al., Brock Biology of Microorganisms, 8th edition, Prentice Hall, UpperSaddle River, NJ (1997)). Facultative methylotrophic bacteria suitable in the present invention include but are not limited to, *Methylophilus*, *Methylobacillus*, *Methylobacterium*, *Hyphomicrobium*, *Xanthobacter*, *Bacillus*, *Paracoccus*, *Nocardia*, *Arthrobacter*, *Rhodopseudomonas*, and *Pseudomonas*.

The ability to utilize single carbon substrates is not limited to bacteria, but also extends to yeasts and fungi. For example a variety of yeast genera are able to use single carbon substrates in addition to more complex materials as energy sources. Specific methylotrophic yeasts useful in the present invention include but are not limited to *Candida*, *Hansenula*, *Pichia*, *Torulopsis*, and *Rhodotorula*.

Those methylotrophs having the additional ability to utilize methane are referred to as methanotrophs. Of interest in the present invention are those obligate methanotrophs which are methane utilizers but which are obliged to use organic compounds lacking carbon-carbon bonds.

Exemplary of these organisms are included in, but not limited to the genera *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylomicrobium*, and *Methanomonas*.

Of particular interest in the present invention are high growth obligate methanotrophs having an energetically favorable carbon flux pathway. For example Applicants have discovered a specific strain of methanotroph having several pathway features which make it particularly useful for carbon flux manipulation. This type of strain has served as the host in present application and is known as *Methylobacter 16a* (ATCC PTA 2402).

The present strain contains several anomalies in the carbon utilization pathway. For example, based on genome sequence data, the strain is shown to contain genes for two pathways of hexose metabolism. The Entner-Doudoroff Pathway which utilizes the keto-deoxy phosphogluconate aldolase enzyme is present in the strain. Is generally well accepted that this is the operative pathway in obligate methanotrophs. Also present, however, is the Embden-Meyerhoff Pathway which utilizes the Fructose biphosphate aldolase enzyme. It is well known that this pathway is either not present or not operative in obligate methanotrophs. Energetically, the latter pathway is most favorable and allows greater yield of biologically useful energy and ultimately production of cell mass and other cell mass-dependent products in *Methylobacter 16a*. The activity of this pathway in the present 16a strain has been confirmed through microarray data and biochemical evidence measuring the reduction of ATP. Although the 16a strain has been shown to possess both the Embden-Meyerhoff and the Entner-Doudoroff pathway enzymes the data suggests that the Embden-Meyerhoff pathway enzymes are more strongly expressed than the Entner-Doudoroff pathway enzymes. This result is surprising and counter to existing beliefs on the glycolytic metabolism of methanotrophic bacteria. Applicants have discovered other methanotrophic bacteria having this characteristic, including for example, *Methylobacter clara* and *Methylobacter sporium*.

A particularly novel and useful feature of the Embden-Meyerhoff pathway in strain 16a is that the key phosphofructokinase step is pyrophosphate dependent instead of ATP dependent. This feature adds to the energy yield of the pathway by using pyrophosphate instead of ATP. Because of its significance in providing an energetic advantage to the strain this gene in the carbon flux pathway is considered diagnostic for the present strain.

utilizing bacterium may have an advantage over other methane utilizing organisms as production platforms for either single cell protein or for any other product derived from the flow of carbon through the RuMP pathway.

Accordingly the present invention provides a method for the

production of a monoterpene compound comprising providing a transformed C1 metabolizing host cell which

(a) grows on a C1 carbon substrate selected from the group consisting of methane and methanol; and

(b) comprises a functional Embden-Meyeroff carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme; and

(c) contains an endogenous source of geranyl diphosphate (GPP)

Isolation of C1 Metabolizing Microorganisms

The C1 metabolizing microorganisms of the present invention are ubiquitous and many have been isolated and characterized. A general scheme for isolation of these strains includes addition of an inoculum into a sealed liquid mineral salts media, containing either methane or methanol. Care must be made of the volume:gas ratio and cultures are typically incubated between 25-55°C. Typically, a variety of different methylotrophic bacteria can be isolated from a first enrichment, if it is plated or streaked onto solid media when growth is first visible. Methods for the isolation of methanotrophs are common and well known in the art (See for example Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA; Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36: 227 (1992); or Hanson, R.S. et al. *The Prokaryotes: a handbook on habitats, isolation, and identification of bacteria*; Springer-Verlag: Berlin, New York, 1981; Volume 2, Chapter 118).

As noted above, preferred C1 metabolizer is one that incorporates an active Embden-Meyerhoff pathway as indicated by the presence of a pyrophosphate dependent phosphofructokinase. It is contemplated that the present teaching will enable the general identification and isolation of similar strains. For example, the key characteristics of the present high growth strain are that it is an obligate methanotroph, using only either methane or methanol as a sole carbon source and possesses a functional Embden-Meyerhoff, and particularly a gene encoding a pyrophosphate

dependent phosphofructokinase. Methods for the isolation of methanotrophs are common and well known in the art (See for example Thomas D. Brock *supra* or Deshpande, *supra*). Similarly, pyrophosphate dependent phosphofructokinase has been well characterized in mammalian systems and assay methods have been well developed (see for example Schliselfeld et al. *Clin. Biochem.* (1996), 29(1), 79-83; Clark et al., *J. Mol. Cell. Cardiol.* (1980), 12(10), 1053-64. The contemporary microbiologist will be able to use these techniques to identify the present high growth strain.

10 Genes involved in Monoterpene synthesis

Many C1 metabolizing strains possess the ability to produce geranyl diphosphate (GPP) which is the substrate for monoterpene synthases. Where a host cell is employed that makes GPP it will only be necessary to introduce the specific terpene synthase for the production of a specific monoterpene.

Many cyclic terpene synthases are known in the art and any one will be suitable for expression in the hosts of the present invention. Limonene synthase is the most well characterized having been isolated from a variety of organisms including *Perilla frutescens* (Genbank Acc #AF317695), *Arabidopsis* (Genbank Acc # AB005235), *Perilla citriodora* (Genbank Acc # AF241790), *Schizonepeta tenuifolia* (Genbank Acc # AF233894), *Abies grandis* (Genbank Acc # AF139207), *Mentha longifolia* (Genbank Acc # AF175323) and *Mentha spicata* (Genbank Acc # L13459). Any one of the known genes encoding limonene synthase may be used for expression in the present invention where genes isolated from *Abies grandis* and *Mentha spicata* are preferred.

Other cyclic terpene synthases are known. For example bornyl diphosphate synthase has been isolated from *Salvia officinalis* (Genbank Acc # AF051900); 1,8-cineole synthase has been isolated from *Salvia officinalis* (Genbank Acc # AF051899); phellandrene synthase has been isolated from *Abies grandis* (Genbank Acc # AF139205); sabinene synthase has been isolated from *Salvia officinalis* (Genbank Acc # AF051901); and pinene synthase has been isolated from *Artemisia annua* (Genbank Acc # AF276072), and *Abies grandis* (Genbank Acc # U87909).

Accordingly, suitable synthases for monoterpene expression include but not limited to limonene synthase, pinene synthase, bornyl

synthase, phellandrene synthase, cineole synthase, and sabinene synthase.

It will be appreciated that where GPP is present in the host cell, expression of a specific terpene synthase will generate the corresponding monoterpene. So for example, the expression of limonene synthase will generate limonene, the expression of pinene synthase will generate pinene, the expression of sabinene synthase will generate sabinene, the expression of phellandrene synthase will generate β -phellandrene and the expression of bornyl diphosphate synthase will generate borneol (Figure 3).

In some instances the specific C1 metabolizing host cell may be lacking some or all the elements of the pathway necessary for the production of geranyl diphosphate (GPP). Alternatively some of the elements of this pathway may be rate limiting and require overexpression for effective synthesis of GPP. In these situations it may be necessary to introduce some or all of the GPP synthetic pathway genes or "upper pathway isoprenoid genes" into the host, or to introduce additional copies of existing genes in the pathway to regulate or increase the production of certain rate limiting steps of the pathway. GPP is the end product of a biosynthetic pathway that begins with the condensation of Glyceraldehyde-3P and pyruvate and ends with the condensation of isopentenyl diphosphate (IPP) and dimethylallyl-diphosphate to form GPP (Figure 3).

Many steps in isoprenoid pathways are known. For example, the initial steps of the alternate pathway involve the condensation of 2 carbons from pyruvate with C1 aldehyde group, D-glyceraldehyde 3-Phosphate to yield 5-carbon compound (D-1-deoxyxylulose-5-phosphate) (Figure 3 and Figure 4). Lois *et al.* has reported a gene, *dxs*, that encodes D-1-deoxyxylulose-5-phosphate synthase (DXS) that catalyzes the synthesis of D-1-deoxyxylulose-5-phosphate in *E. coli* (*Proc. Natl. Acad. Sci. USA* 95: 2105-2110 (1998)).

Next, the isomerization and reduction of D-1-deoxyxylulose-5-phosphate yields 2-C-methyl-D-erythritol-4-phosphate. One of the enzymes involved in the isomerization and reduction process is D-1-deoxyxylulose-5-phosphate reductoisomerase (DXR). Takahashi *et al.* reported that *dxr* gene product catalyzes the formation of 2-C-methyl-D-

erythritol-4-phosphate in the alternate pathway in *E. coli* (*Proc. Natl. Acad. Sci. USA* 95: 9879-9884 (1998)).

Steps converting 2-C-methyl-D-erythritol-4-phosphate to isopentenyl monophosphate are not well characterized although some steps are known. 2-C-methyl-D-erythritol-4-phosphate is then converted into 4-diphosphocytidyl-2C-methyl-D-erythritol in a CTP dependent reaction by the enzyme encoded by non-annotated gene *ygbP*. Rohdich *et al.* reported YgbP, a protein in *E. coli* that catalyzes the reaction mentioned above. Recently, *ygbP* gene was renamed as *ispD* as a part of *isp* gene cluster (SwissProt#Q46893) (*Proc. Natl. Acad. Sci. USA* 96:11758-11763 (1999)).

Then the 2 position hydroxy group of 4-diphosphocytidyl-2C-methyl-D-erythritol can be phosphorylated in an ATP dependent reaction by the enzyme encoded by *ychB* gene. Luttgen *et al.* has reported the presence of YchB protein in *E. coli* that phosphorylates 4-diphosphocytidyl-2C-methyl-D-erythritol resulting in 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate. Recently, *ychB* gene was renamed as *ispE* as a part of *isp* gene cluster (SwissProt #P24209) (Luttgen *et al.*, *Proc. Natl. Acad. Sci. USA* 97:1062-1067 (2000)).

Herz *et al.* reported that *ygbB* gene product in *E. coli* converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate. 2C-methyl-D-erythritol 2,4-cyclodiphosphate can be further converted into carotenoids in the carotenoid biosynthesis pathway (*Proc. Natl. Acad. Sci. USA* 97:2486-2490 (2000)). Recently, *ygbB* gene was renamed as *ispF* as a part of *isp* gene cluster (SwissProt #P36663).

The reaction catalyzed by YgbP enzyme is carried out in CTP dependent manner. Thus CTP synthase plays an important role in the isoprenoid pathway. PyrG encoded by *pyrG* gene in *E. coli* was determined to encode CTP synthase (Weng *et al.*, *J. Biol. Chem.*, 261:5568-5574 (1986)).

Followed by the reactions not yet characterized, isopentenyl monophosphate is formed. Isopentenyl monophosphate is converted to isopentenyl diphosphate (IPP, C5) by isopentenyl monophosphate kinase encoded by *ipk* gene that is identical to the above mentioned *yhcB* (*ispE*) gene (Lange and Croteau, *Proc. Natl. Acad. Sci. USA* 96:13714-13719 (1999)). Isopentenyl diphosphate (IPP) is isomerized to dimethylallyl-

pyrophosphate (DMAPP) by IPP:DMAPP isomerase (IPP isomerase, EC 5.3.3.2) or isopentenyl diphosphate isomerase (idi). Alternatively, recent evidence suggests that DMAPP can be formed separately at an earlier step of the mevalonate-independent pathway (Cunningham *et al*, *J. Bac.* 182 No. 20: 5841-5848(2000)), and that the enzyme encoded by *lytB* plays an essential role for this alternate route of DMAPP formation. DMAPP and IPP are condensed by geranyltranstransferase (*ispA*) gene (Ohto *et al.* *Plant Mol. Biol.* 40 (2), 307-321 (1999) to produce the linear C-10 compound geranyl diphosphate (GPP) which is the substrate for monoterpene synthases.

Accordingly, where it is necessary to regulate or install elements of the pathway needed for the synthesis of GPP in any C1 metabolizer, genes, known in the art, encoding the enzymes selected the group consisting of Dxs (1-deoxyxylulose-5-phosphate synthase), Dxr (1-deoxyxylulose-5-phosphate reductoisomerase), *IspD* (2C-methyl-D-erythritol cytidyltransferase), *IspE* (4-diphosphocytidyl-2-C-methylerythritol kinase), *IspF*, (2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase), *PyrG* (CTP synthase), *IspA* (Geranyltransferase or farnesyl diphosphate synthase) and *LytB* may be used in the present C1 metabolizer host cell.

Construction of a Recombinant C1 Metabolizer for Monoterpene Production

Methods for introduction of genes encoding the appropriate cyclic terpene synthase into a suitable methylotrophic host are common. Microbial expression systems and expression vectors containing regulatory sequences suitable for expression of heterologous genes in methylotrophs are known. Any of these could be used to construct chimeric genes for expression of the any of the above mentioned cyclic terpene synthases. These chimeric genes could then be introduced into appropriate methylotrophic hosts via transformation to provide high level expression of the enzymes.

Vectors or cassettes useful for the transformation of suitable host cells are available. For example several classes of promoters may be used for the expression of genes encoding cyclic terpene synthases in methylotrophs and methanotrophs including, but not limited to endogenous promoters such as the deoxy-xylulose phosphate synthase, methanol dehydrogenase operon promoter (Springer *et al.* (1998) *FEMS Microbiol Lett* 160:119-124) the promoter for polyhydroxyalkanoic acid

synthesis (Foellner et al. *Appl. Microbiol. Biotechnol.* (1993) 40:284-291), or promoters identified from native plasmid in methylotrophs (EP 296484) In addition to these native promoters non-native promoters may also be used, as for example the the promoter for lactose operon *Plac* (Toyama et al. *Microbiology* (1997) 143:595-602; EP 62971) or a hybrid promoter such as *P_{trc}* (Brosius et al. (1984) *Gene* 27:161-172). Similarly promoters associated with antibiotic resistance e.g. kanamycin (Springer et al. (1998) *FEMS Microbiol Lett* 160:119-124; Ueda et al. *Appl. Environ. Microbiol.* (1991) 57:924-926) or tetracycline (US 4824786) are also suitable.

Once the specific regulatory element is selected the promoter-gene cassette can be introduced into methylotrophs on a plasmid containing either a replicon (Brenner et al. *Antonie Van Leeuwenhoek* (1991) 60:43-48; Ueda et al. *Appl. Environ. Microbiol.* (1991) 57:924-926) for episomal expression or homologous regions for chromosomal integration (Naumov et al. *Mol. Genet. Mikrobiol. Virusol.* (1986) 11:44-48).

Typically the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are derived from genes homologous to the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

Where accumulation of a specific monoterpene is desired it may be necessary to reduce or eliminate the expression of certain genes in the target pathway or in competing pathways that may serve as competing sinks for energy or carbon. Methods of down-regulating genes for this purpose have been explored. Where sequence of the gene to be disrupted is known, one of the most effective methods gene down regulation is targeted gene disruption where foreign DNA is inserted into a structural gene so as to disrupt transcription. This can be effected by the creation of genetic cassettes comprising the DNA to be inserted (often a genetic marker) flanked by sequence having a high degree of homology to a portion of the gene to be disrupted. Introduction of the cassette into the host cell results in insertion of the foreign DNA into the structural gene via

the native DNA replication mechanisms of the cell. (See for example Hamilton et al. (1989) *J. Bacteriol.* 171:4617-4622, Balbas et al. (1993) *Gene* 136:211-213, Gueldener et al. (1996) *Nucleic Acids Res.* 24:2519-2524, and Smith et al. (1996) *Methods Mol. Cell. Biol.* 5:270-277.)

5 Antisense technology is another method of down regulating genes where the sequence of the target gene is known. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the anti-sense strand of RNA will be transcribed. This construct is then introduced into the host cell and the antisense strand
10 of RNA is produced. Antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the protein of interest. The person skilled in the art will know that special considerations are associated with the use of antisense technologies in order to reduce expression of particular genes. For example, the proper level of
15 expression of antisense genes may require the use of different chimeric genes utilizing different regulatory elements known to the skilled artisan.

Although targeted gene disruption and antisense technology offer effective means of down regulating genes where the sequence is known, other less specific methodologies have been developed that are not
20 sequence based. For example, cells may be exposed to a UV radiation and then screened for the desired phenotype. Mutagenesis with chemical agents is also effective for generating mutants and commonly used substances include chemicals that affect non-replicating DNA such as HNO₂ and NH₂OH, as well as agents that affect replicating DNA such as
25 acridine dyes, notable for causing frameshift mutations. Specific methods for creating mutants using radiation or chemical agents are well documented in the art. See for example Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund
30 V., *Appl. Biochem. Biotechnol.*, 36, 227, (1992).

Another non-specific method of gene disruption is the use of transposable elements or transposons. Transposons are genetic elements that insert randomly in DNA but can be latter retrieved on the basis of sequence to determine where the insertion has occurred. Both
35 *in vivo* and *in vitro* transposition methods are known. Both methods involve the use of a transposable element in combination with a transposase enzyme. When the transposable element or transposon, is contacted with

a nucleic acid fragment in the presence of the transposase, the transposable element will randomly insert into the nucleic acid fragment. The technique is useful for random mutagenesis and for gene isolation, since the disrupted gene may be identified on the basis of the sequence of the transposable element. Kits for *in vitro* transposition are commercially available (see for example The Primer Island Transposition Kit, available from Perkin Elmer Applied Biosystems, Branchburg, NJ, based upon the yeast Ty1 element; The Genome Priming System, available from New England Biolabs, Beverly, MA; based upon the bacterial transposon Tn7; and the EZ::TN Transposon Insertion Systems, available from Epicentre Technologies, Madison, WI, based upon the Tn5 bacterial transposable element).

In the context of the present invention the disruption of certain genes in the terpenoid pathway may enhance the accumulation of specific monoterpenes however, the decision of which genes to disrupt would need to be determined on an empirical basis. Candidate genes may include one or more of the prenyltransferase genes which, as described earlier, which catalyze the successive condensation of isopentenyl diphosphate resulting in the formation of prenyl diphosphates of various chain lengths (multiples of C-5 isoprene units). Other candidate genes for disruption would include any of those which encode proteins acting upon the terpenoid backbone prenyl diphosphates.

Industrial Production

Where commercial production of the instant proteins are desired a variety of culture methodologies may be applied. For example, large-scale production of a specific gene product, overexpressed from a recombinant microbial host may be produced by both batch or continuous culture methodologies.

A classical batch culturing method is a closed system where the composition of the media is set at the beginning of the culture and not subject to artificial alterations during the culturing process. Thus, at the beginning of the culturing process the media is inoculated with the desired organism or organisms and growth or metabolic activity is permitted to occur adding nothing to the system. Typically, however, a "batch" culture is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system

change constantly up to the time the culture is terminated. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase are often responsible for the bulk of production of end product or intermediate in some systems. Stationary or post-exponential phase production can be obtained in other systems.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch culture processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the culture progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and Fed-Batch culturing methods are common and well known in the art and examples may be found in Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36, 227, (1992), herein incorporated by reference.

Commercial production of the instant proteins may also be accomplished with a continuous culture. Continuous cultures are an open system where a defined culture media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous cultures generally maintain the cells at a constant high liquid phase density where cells are primarily in log phase growth. Alternatively continuous culture may be practiced with immobilized cells where carbon and nutrients are continuously added, and valuable products, by-products or waste products are continuously removed from the cell mass. Cell immobilization may be performed using a wide range of solid supports composed of natural and/or synthetic materials.

Continuous or semi-continuous culture allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient

such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the culture. Methods of modulating nutrients and growth factors for continuous culture processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

Fermentation media in the present invention must contain suitable carbon substrates. The suitable carbon substrate may be one-carbon substrates such as methane or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., *Microb. Growth C1 Compd.*, [Int. Symp.], 7th (1993), 415-32. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter et al., *Arch. Microbiol.* 153:485-489 (1990)). Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism.

Description of the Preferred Embodiments

Methylomonas 16a was isolated from a pond sediment using methane as sole source of carbon and energy. Among the colonies that were able to grow using methane as a sole source of carbon and energy, *Methylomonas* 16a strain was chosen for its rapid growth rate and pink pigmentation indicating inherent isoprenoid pathway for carotenoids.

The carbon flux pathways in *Methylomonas* 16a were analyzed by gene expression profiling and the presence and activity of the Embden-Meyerhoff pathway, comprising the presence of a functional pyrophosphate-linked phosphofructokinase enzyme as confirmed.

A truncated limonene synthase gene lacking the first 57 amino acids of the protein from *Mentha spicata* was obtained from pR58 plasmid. The truncated limonene synthase gene was cloned into the broad host vector pTJS75:dxS:dxR:Tn5Kn. The resulting plasmid pDH3 was transferred into *Methylomonas* 16a by triparental conjugal mating with

fresh overnight cultures of *E. coli* helper pRK2013 and *E. coli* donor DH10B/pDH3.-Vector pTJS75:dxS:dxR:Tn5Kn was similarly transferred into *Methylobionas*. Cloning methods and triparental conjugal mating are well known in the art. The presence of limonene synthase gene is verified using PCR.

The transformed culture of *Methylobionas* 16a was grown in airtight bottles to prevent the loss of volatile limonene compound. The compound produced by transformed *Methylobionas* 16a was extracted and analyzed by gas chromatography. The compound was confirmed to be limonene when compared to standard limonene. Approximately 0.5ppm of limonene was detected from transformed culture.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, (1989) (Maniatis) and by T. J. Silhavy, M. L. Bannan, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in *Manual of Methods for General Bacteriology* (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994)) or by Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, MA

(1989). All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless
5 otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

Microbial Cultivation and Preparation of Cell Suspensions, and associated
10 analyses.

Methylobomonas 16a is typically grown in serum stoppered Wheaton bottles using a gas/liquid ratio of at least 8:1 (i.e. 20 mL of Nitrate liquid media) media in a Wheaton bottle (Wheaton Scientific, Wheaton IL) of 160 mL total volume. The standard gas phase for cultivation contained
15 25% methane in air. These conditions comprise growth conditions and the cells are referred to as growing cells. In all cases the cultures were grown at 30°C with constant shaking in a Lab-Line rotary shaker unless otherwise specified.

Cells obtained for experimental purposes were allowed to grow to
20 maximum optical density (O.D. 660 ~ 1.0). Harvested cells were obtained by centrifugation in a Sorval RC-5B centrifuge using a SS-34 rotor at 6000 rpm for 20 min. These cell pellets were resuspended in 50 mM HEPES buffer pH 7. These cell suspensions are referred to as washed, resting cells.

25 Microbial growth was assessed in all experiments by measuring the optical density of the culture at 660 nm in an Ultrospec 2000 UV/Vis spectrophotometer (Pharmacia Biotech, Cambridge England) using a 1 cm light path cuvet. Alternatively microbial growth was assessed by harvesting cells from the culture medium by centrifugation as described above and
30 resuspending the cells in distilled water with a second centrifugation to remove medium salts. The washed cells were then dried at 105°C overnight in a drying oven for dry weight determination.

Methane concentration was determined as described by Emptage et al. (1997 *Env. Sci. Technol.* 31:732-734), hereby incorporated by
35 reference.

Nitrate medium for *Methylomonas* 16A

Nitrate liquid medium, also referred to herein as “defined medium” was comprised of various salts mixed with solution 1 as indicated below or where specified the nitrate was replaced with 15 mM ammonium chloride.

- 5 Solution 1 Composition for 100 fold concentrated stock solution of trace minerals.

	MW	Conc. (mM)	g per L
Nitriloacetic acid	191.1	66.9	12.8
CuCl ₂ x 2H ₂ O	170.48	0.15	0.0254
FeCl ₂ x 4H ₂ O	198.81	1.5	0.3
MnCl ₂ x 4H ₂ O	197.91	0.5	0.1
CoCl ₂ x 6H ₂ O	237.9	1.31	0.312
ZnCl ₂	136.29	0.73	0.1
H ₃ BO ₃	61.83	0.16	0.01
Na ₂ MoO ₄ x 2H ₂ O	241.95	0.04	0.01
NiCl ₂ x 6H ₂ O	237.7	0.77	0.184

- 10 Mix the gram amounts designated above in 900 mL of H₂O, adjust to pH=7, and add H₂O to an end volume of 1 L. Keep refrigerated.

Nitrate liquid medium:

	MW	Conc. (mM)	g per L
NaNO ₃	84.99	10	0.85
KH ₂ PO ₄	136.09	3.67	0.5
Na ₂ SO ₄	142.04	3.52	0.5
MgCl ₂ x 6H ₂ O	203.3	0.98	0.2
CaCl ₂ x 2H ₂ O	147.02	0.68	0.1
1 M HEPES (pH 7)	238.3		50 mL
Solution 1			10 mL

- 15 Dissolve in 900 mL H₂O. Adjust to pH=7, and add H₂O to give 1 L.
For agar plates: Add 15 g of agarose in 1 L of medium, autoclave, let cool down to 50°C, mix, and pour plates.

Nitrate and Nitrite Assays

1 mL samples of cell culture were taken and filtered through a 0.2 micron Acrodisc filter to remove cells. The filtrate from this step contains the nitrite or nitrate to be analyzed. The analysis was performed on a Dionex ion chromatograph 500 system (Dionex, Sunnyvale, CA) with an AS3500 autosampler. The column used was a 4 mm Ion-Pac AS11-HC separation column with an AG-AC guard column and an ATC trap column. All columns are provided by Dionex.

The mobile phase was a potassium hydroxide gradient from 0 to 50 mM potassium hydroxide over a 12 min time interval. Cell temperature was 35°C with a flow rate of 1 mL/min.

Microarray of gene expression

Amplification of DNA regions for the construction of DNA microarray: Specific primer pairs were used to amplify each protein specifying ORF of *Methylomonas* sp. strain 16a. Genomic DNA (10-30 ng) was used as the template. The PCR reactions were performed in the presence of HotStart Taq™ DNA polymerase (Qiagen, Valencia, CA) and the dNTPs (Gibco BRL Life Science Technologies, Gaithersburg, MD). Thirty-five cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and polymerization at 72°C for 2 min were conducted. The quality of PCR reactions was checked with electrophoresis in a 1% agarose gel. The DNA samples were purified by the high-throughput PCR purification kit from Qiagen.

Arraying amplified ORFs. Before arraying, an equal volume of DMSO (10 µL) and DNA (10 µL) sample was mixed in 384-well microtiter plates. A generation II DNA spotter (Molecular Dynamics, Sunnyvale, CA) was used to array the samples onto coated glass slides (Telechem, Sunnyvale, CA). Each PCR product was arrayed in duplicate on each slide. After cross-linking by UV light, the slides were stored under vacuum in a desiccator at room temperature.

RNA isolation: *Methylomonas* 16a was cultured in a defined medium with ammonium or nitrate (10 mM) as nitrogen source under 25% methane in air. Samples of the minimal medium culture were harvested when the O.D. reaches 0.3 at A₆₀₀ (exponential phase). Cell cultures were harvested quickly and ruptured in RLT buffer [Qiagen RNeasy Mini Kit, Valencia, CA] with a beads-beater (Bio101, Vista, CA). Debris was pelleted by centrifugation for 3 min at 14,000 x g at 4°C. RNA isolation was completed using the protocol supplied with this kit. After on-column

DNAase treatment, the RNA product was eluted with 50-100 μ L RNAase-free. RNA preparations were stored frozen at either -20 or -80°C.

5 Synthesis of fluorescent cDNA from total RNA. RNA samples (7 to 15 μ g) and random hexamer primers (6 μ g; Gibco BRL Life Science Technologies) were diluted with RNAase-free water to a volume of 25 μ L. The sample was denatured at 70°C for 10 min and then chilled on ice for 30 seconds. After adding 14 μ L of labeling mixture, the annealing was accomplished by incubation at room temperature for 10 min. The labeling mixture contained 8 μ L of 5x enzyme buffer, 4 μ L DTT (0.1M), and 2 μ L of 10 20x dye mixture. The dye mixture consisted of 2 mM of each dATP, dGTP, and dTTP, 1 mM dCTP, and 1 mM of Cy3-dCTP or Cy5-dCTP. After adding 1 to 1.5 μ L of SuperScript II reverse transcriptase (200 units/mL, Life Technologies Inc., Gaithersburg, MD), cDNA synthesis was allowed to proceed at 42°C for 2 hr. The RNA was removed by 15 adding 2 μ L NaOH (2.5 N) to the reaction. After 10 min of incubation at 37°C, the pH was adjusted with 10 μ L of HEPES (2M). The labeled cDNA was then purified with a PCR purification kit (Qiagen, Valencia, CA). Labeling efficiency was monitored using either A₅₅₀ for Cy3 incorporation, or A₆₅₀ for Cy5.

20 Fluorescent labeling of genomic DNA. Genomic DNA was nebulized to approximately 2 kb pair fragments. Genomic DNA (0.5 to 1 μ g) was mixed with 6 μ g of random hexamers primers (Gibco BRL Life Science Technologies) in 15 μ L of water. The mix was denatured by put at boiling water for 5 minutes. Then anneal on ice for 30 sec before put at 25 room temperature. Then 2 μ L 5x Buffer 2 (Gibco BRL) and 2 μ L dye mixture were added. The component of dye mixture and the labeling procedure are the same as described above for RNA labeling, except that the Klenow fragment of DNA polymerase I (5 μ g/ μ L, Gibco BRL Life Science Technologies) was used as the enzyme. After incubation 37 °C 30 for 2 hr, the labeled DNA probe was purified using a PCR purification kit (Qiagen, Valencia, CA).

35 Hybridization and washing. Slides were first incubated with prehybridization solution containing 3.5xSSC (BRL, Life Technologies Inc., Gaithersburg, MD), 0.1% SDS (BRL, Life Technologies Inc., Gaithersburg, MD), 1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO). After prehybridization, hybridization solutions (Molecular Dynamics) containing labeled probes was added to slides and covered with cover

slips. Slides were placed in a humidified chamber in a 42°C incubator. After overnight hybridization, slides were initially washed for 5 min at room temperature with a washing solution containing 1xSSC, 0.1% SDS and 0.1xSSC, 0.1% SDS. Slides were then washed at 65°C for 10 min with the same solution for three times. After washing, the slides were dried with a stream of nitrogen gas.

Data Collection and Analysis. The signal generated from each slide was quantified with a laser scanner (Molecular Dynamics, Sunnyvale, CA). The images were analyzed with ArrayVision 4.0 software (Imaging Research, Inc., Ontario, Canada). The raw fluorescent intensity for each spot was adjusted by subtracting the background. These readings were exported to a spreadsheet for further analysis.

EXAMPLE 1

ISOLATION OF METHYLOMONAS 16A

The original environmental sample containing the isolate was obtained from pond sediment. The pond sediment was inoculated directly into defined medium with ammonium as nitrogen source under 25% methane in air. Methane was the sole source of carbon and energy. Growth was followed until the optical density at 660 nm was stable whereupon the culture was transferred to fresh medium such that a 1:100 dilution was achieved. After 3 successive transfers with methane as sole carbon and energy source the culture was plated onto growth agar with ammonium as nitrogen source and incubated under 25% methane in air. Many methanotrophic bacterial species were isolated in this manner. However, *Methylomonas* 16a was selected as the organism to study due to the rapid growth of colonies, large colony size, ability to grow on minimal media, and pink pigmentation indicative of an active biosynthetic pathway for carotenoids.

EXAMPLE 2

RAPID GROWTH ON METHANE IN MINIMAL MEDIUM

Methylomonas 16a grows on the defined medium comprised of only minimal salts, a culture headspace comprised of methane in air. Methane concentrations for growth but typically are 5-50% by volume of the culture headspace. No organic additions such as yeast extract or vitamins are required to achieve growth shown in Figure 5. Figure 5 shows the growth of 16a compared to the growth of *Methylococcus capsulatus* under identical growth conditions. i.e. minimal medium with 25% methane in air

as substrate. The data indicates *Methylobacter* 16a doubles every 2-2.5 h whereas *Methylobacter capsulatus* doubles every 3.5 h with methane as substrate. With methanol as substrate doubling times on methanol are 2.5-3 for *Methylobacter* 16a and 4.5-5 for *Methylobacter capsulatus*. Cell densities are also significantly higher for *Methylobacter* 16a growing on methane. *Methylobacter capsulatus* is a widely utilized methanotroph for experimental and commercial purposes.

EXAMPLE 3

COMPARISON OF GENE EXPRESSION LEVELS IN THE ENTNER DOUDEROFF PATHWAY AS COMPARED WITH THE EMBDEN MEYEROF PATHWAY

Example 3 presents microarray evidence for the use of the Embden-Meyerhoff pathway in the 16a strain.

Figure 6 shows the relative levels of expression of genes for the Entner-Douderooff pathway and the Embden-Meyerhof pathway. The relative transcriptional activity of each gene was estimated with DNA microarray as described previously (Wei, *et al.*, 2001. *Journal of Bacteriology*. 183:545-556).

Specifically, a single DNA microarray containing 4000 ORFs (open reading frames) of *Methylobacter* sp. strain 16a was hybridized with probes generated from genomic DNA and total RNA. The genomic DNA of 16a was labeled with Klenow fragment of DNA polymerase and fluorescent dye Cy-5, while the total RNA was labeled with reverse transcriptase and Cy-3. After hybridization, the signal intensities of both Cy-3 and Cy-5 for each spot in the array were quantified. The intensity ratio of Cy-3 and Cy-5 was then used to calculate the fraction of each transcript (in percentage) with the following formula: (gene ratio/sum of all ratio) x 100. The value obtained reflects the relative abundance of mRNA of an individual gene. Accordingly, transcriptional activity of all the genes represented by the array can be ranked based on its relative mRNA abundance in a descending order. For example, mRNA abundance for the methane monooxygenase was ranked #1 because its genes had the highest transcriptional activity when the organism was grown with methane as the carbon source (Figure 6).

The genes considered "diagnostic" for Entner-Douderooff are the 6 phosphogluconate dehydratase and the 2 keto-3-deoxy-6-phosphogluconate aldolase. Phosphofructokinase and fructose

bisphosphate aldolase are "diagnostic" of the Embden-Meyerhof sequence. Numbers in Figure 6 next to each step indicate the relative expression level of that enzyme. For example the most highly expressed enzyme in the cell is the methane monooxygenase (ranked #1). The next most highly expressed is the methanol dehydrogenase (ranked #2). Messenger RNA transcripts of Phosphofructokinase (ranked #232) and fructose bisphosphate aldolase (ranked #65) were in higher abundance than those for glucose 6 phosphate dehydrogenase (ranked #717), 6 phosphogluconate dehydratase (ranked #763) or the 2-keto-3-deoxy-6-gluconate aldolase. The data suggests that the Embden-Meyerhof pathway enzymes are more strongly expressed than the Entner-Doudoroff pathway enzymes. This result is surprising and counter to existing beliefs on the central metabolism of methanotrophic bacteria (Reference book pages in. The physiology and biochemistry of aerobic methanol-utilizing gram-negative and gram- positive bacteria In: Methane and Methanol Utilizers, Biotechnology Handbooks 5. 1992. Eds: Colin Murrell, Howard Dalton. Pp. 149-157.

EXAMPLE 4

DIRECT ENZYMATIC EVIDENCE FOR A PYROPHOSPHATE-LINKED PHOSPHOFRUCTOKINASE

Example 4 shows the evidence for the presence of a pyrophosphate-linked phosphofructokinase enzyme in the current strain which would confirm the functionality of the Embden-Meyerhof pathway in the present strain.

Phosphofructokinase activity was shown to be present in *Methylobacter* 16a by using the coupled enzyme assay described below. Assay conditions are given in Table 2 below. This assay was further used to assay the activity in a number of other Methanotrophic bacteria as shown below in Table 3. The data in Table 3 show known ATCC strains tested for phosphofructokinase activity with ATP or pyrophosphate as phosphoryl donor. These organisms were classified as either Type I or Type X ribulose monophosphate-utilizing strains or Type II serine utilizer.

Coupled Assay Reactions

Phosphofructokinase reaction was measured by a coupled enzyme assay. Phosphofructokinase reaction was coupled with fructose 1,6, biphosphate aldolase followed by triosephosphate isomerase. The enzyme activity was measured by the disappearance of NADH.

Specifically, the enzyme phosphofructokinase catalyzes the key reaction converting Fructose 6 phosphate and pyrophosphate to Fructose 1,6 bisphosphate and orthophosphate. Fructose-1,6-bisphosphate is cleaved to 3-phosphoglyceraldehyde and dihydroxyacetonephosphate by fructose 1,6-bisphosphate aldolase. Dihydroxyacetonephosphate is isomerized to 3-phosphoglyceraldehyde by triosephosphate isomerase. Glycerol phosphate dehydrogenase plus NADH and 3-phosphoglyceraldehyde yields the alcohol glycerol-3-phosphate and NAD. Disappearance of NADH is monitored at 340 nm using spectrophotometer (UltraSpec 4000, Pharmacia Biotech).

Table 2
Assay Protocol

Reagent	Stock solution (mM)	Volume (μ l) per 1mL total reaction volume	Final assay concentration (mM)
Tris-HCl pH 7.5	1000	100	100
MgCl ₂ . 2 H ₂ O	100	35	3.5
Na ₄ P ₂ O ₇ .10H ₂ O or ATP	100	20	2
Fructose-6-phosphate	100	20	2
NADH	50	6	0.3
Fructose bisphosphate aldolase	100 (units/mL)	20	2 (units)
Triose phosphate isomerase/glyceral phosphate dehydrogenase	(7.2 units/ μ l) (0.5 units/ μ l)	3.69	27 units 1.8 units
KCl	1000	50	50
H ₂ O		adjust to 1mL	
Crude extract		0-50	

Table 3
Comparison Of Pyrophosphate Linked And ATP Linked
Phosphofructokinase Activity In Different Methanotrophic Bacteria

Strain	Type	Assimilation Pathway	ATP-PFK umol NADH/ min/mg	Ppi-PFK umol NADH/ min/mg
Methylomonas 16a ATCC PTA 2402	I	Ribulose monophosphate	0	2.8
Methylomonas agile ATCC 35068	I	Ribulose monophosphate	0.01	3.5
Methylobacter Whittenbury ATCC 51738	I	Ribulose monophosphate	0.01	0.025
Methylomonas clara ATCC 31226	I	Ribulose monophosphate	0	0.3
Methylomicrobium albus ATCC 33003	I	Ribulose monophosphate	0.02	3.6
Methylococcus capsulatus ATCC 19069	X	Ribulose monophosphate	0.01	0.04
Methylosinus sporium ATCC 35069	II	Serine	0.07	0.4

Several conclusions may be drawn from the data presented above. First, it is clear that ATP (which is the typical phosphoryl donor for phosphofructokinase) is essentially ineffective in the phosphofructokinase reaction in methanotrophic bacteria. Only inorganic pyrophosphate was found to support the reaction in all methanotrophs tested. Secondly not all methanotrophs contain this activity. The activity was essentially absent in *Methylobacter whittenbury* and in *Methylococcus capsulatus*. Intermediate levels of activity were found in *Methylomonas clara* and *Methylosinus sporium*. These data show that many methanotrophic bacteria may contain a hitherto unreported phosphofructokinase activity. It may be inferred from this that methanotrophs containing this activity have an active Embden-Meyerhof pathway.

EXAMPLE 5 GROWTH YIELD AND CARBON CONVERSION BY METHYLOMONAS

16A

Growth yield and carbon conversion efficiency were compared for *Methylomonas 16a* and *Methylococcus capsulatus*. These strains were

chosen because 16a contains high levels of phosphofructokinase and *M. capsulatus* is essentially devoid of the enzyme activity. It was contemplated that if *Methylomonas* 16a could utilize the more energetically favorable Embden-Meyerhof pathway and *Methylococcus capsulatus* could only use the Entner-Doudoroff pathway the superior energetics of the present *Methylomonas* 16a strain would be reflected in cellular yields and carbon conversion efficiency. This difference in energetic efficiency would only be apparent under energy-limiting conditions. These conditions were achieved in this experiment by limiting the amount of oxygen in each culture to only 10% (vol/vol) instead of 20%. Under these oxygen limiting conditions the strain that produces the most energy from aerobic respiration on methane will produce more cell mass.

Cells were grown as 200 mL cultures 500 mL serum-stoppered Wheaton bottles. The headspace in the bottles was adjusted to 25% methane and 10% oxygen. The defined medium formulation is the same in both cases.

Table 4
Yield Of Methylomonas 16a Cells Versus Methylococcus Capsulatus Cells
Under Oxygen Limitation.

Strain	Y_{CH_4} g dry wt/mol	G dry wt/g CH_4	Carbon Conversion Efficiency (CCE)%
<i>Methylomonas</i> 16a	16.7 +/- 0.5	1.04	64
<i>Methylococcus capsulatus</i>	10.3 +/- 0.3	0.64	40

Yield determination: Yield was measured by growing triplicate cultures in 500 mL bottles on defined medium with ammonium as nitrogen source under oxygen limitation. This was done by using 300 mL of culture with a 300 mL headspace of 25% methane and 10% oxygen the balance being nitrogen. At the end of growth (i.e. stationary phase) residual methane in the headspace was determined by gas chromatography. The cells were collected by centrifugation washed with distilled water and dried overnight in a drying oven before being weighed.

Carbon conversion efficiency is a measure of how much carbon is assimilated into cell mass. It is calculated assuming a biomass composition of $\text{CH}_2\text{O}_{0.5}\text{N}_{0.25}$:

5 *Methylomonas* 16a : 16 g/mol methane X (1 g dry wt/g methane) /
25 g/ mol biomass

M. capsulatus 16 g/mol methane X (0.64 g dry wt/g methane) /
25 g / mol biomass

 These data (in Table 4) show that *Methylomonas* 16a produced significantly more cell mass than did the *Methylococcus capsulatus* strain under growth conditions that were identical except for the temperature.
10 *Methylococcus capsulatus* grows optimally at 45°C whereas *Methylomonas* is grown at 33°C. It may be inferred from the data that the presence of the more energy-yielding Embden-Meyerhof pathway confers a growth advantage to *Methylomonas* 16a.

15 Table 5 presents the theoretical calculations showing ATP yield as a function of carbon assimilation pathway with the carbon output being normalized to pyruvate in all cases (The physiology and biochemistry of aerobic methanol-utilizing gram-negative and gram- positive bacteria In: Methane and Methanol Utilizers, Biotechnology Handbooks 5. 1992. Eds: Colin Murrell, Howard Dalton. Pp. 149-157). Table 5 shows the amount of
20 ATP that is produced or consumed for every three molecules of carbon (as formaldehyde or carbon dioxide) for serine cycle, xylulose monophosphate cycle and ribulose monophosphate cycle pathways. The latter pathway, as discussed is typically thought to exist as the 2-keto-3deoxy-6-
25 phosphogluconate /transaldolase (KDPGA/TA) variant. These data shows that in fact the fructose bisphosphate aldolase/transaldolase (FBPA/TA) variant is likely to exist in the methanotrophs. The energetic repercussion of this is the net production of an additional 1 ATP for methanotrophs if they possess an ATP linked phosphofructokinase and an additional 2 ATPs
30 for the pyrophosphate-linked enzyme. It is therefore expected that *Methylomonas* 16a derives and additional 2 ATP per 3 carbons assimilated and that this may explain the greater yield and carbon efficiency of the strain versus *Methylococcus capsulatus*.

Table 5
Energetics of Methanotrophic bacteria utilizing different carbon
assimilation mechanisms

Organism	Cycle	C1 unit fixed	Product	Variant	ATP	NADPH
Bacteria	RuMP	3CH ₂ O	Pyruvate	FBPA/TA	+1	+1
Methylomonas	RuMP /Serine	3CH ₂ O	Pyruvate	FBPA/TA	+1(+2*)	+1
Bacteria	RuMP	3CH ₂ O	Pyruvate	KDPGA/TA	0	+1
Methylococcus	RuMP/RuBP	3CH ₂ O	Pyruvate	KDPGA/TA	0	+1

5 * Based on PPi dependent phosphofructokinase

EXAMPLE 6 Plasmid Construction.

10 The plasmid pR58 contains *Mentha spicata* limonene synthase
gene which carries a deletion of the first 57 amino acids of the enzyme
(Williams *et al*, *Biochemistry* 1998, 37, 12213-12220). pR58 was digested
with restriction enzymes *NdeI* and *BamHI*, releasing the truncated
limonene synthase. The 1638 base pair gene was purified by agarose gel
electrophoresis and QIAEX II Gel Extraction (Qiagen Cat.# 20021). The
15 4 base pair 5' overhang (5'-TATG-3') gap was filled using T4 DNA
polymerase (Gibco BRL Cat.# 18005-017), maintaining the methionene
start codon immediately preceding the arginine codon which corresponds
to amino acid number 58 in the native gene. Concurrently, the 3' *BamHI*
overhang was filled in by the same enzyme without interruption of the
20 native limonene synthase stop codon. The blunt ended insert was purified
using the QIAquick PCR Purification Kit (Qiagen Cat# 28104).

25 The vector pTJS75:dxS:dxR:Tn5Kn is a derivative of RK2, a broad-
host-range plasmid (*J. Bact.*, 164, 446-455) modified to include kanamycin
resistance and two *Methylomonas* genes of interest: *dxs*, encoding
5'deoxy-D-xylulose synthase and *dxr*, encoding 5'deoxy-D-xylulose
reductoisomerase. This vector was prepared for ligation by digesting with
XhoI, blunting the overhangs with T4 DNA Polymerase, dephosphorylating
with Calf Intestinal Alkaline Phosphatase (GibcoBRL), and purifying on a
QIAquick PCR Purification column.

30 The limonene synthase gene insert was ligated into the vector and
electroporated into *E. coli* electroMAX DH10B cells (Gibco BRL). The

resulting plasmid, designated pDH3, contains the limonene synthase gene flanked on the 5'end by *dxS* and on the 3'end by *dxR* (Figure 1).

Plasmid pDH3 was transferred into *Methylomonas sp. 16a* by triparental conjugal mating. Fresh overnight cultures of *E. coli* helper pRK2013 and *E. coli* donor DH10B/pDH3 along with vector (pTJS75:dxS:dxR:Tn5Kn) control grown in LB (Luria-Bertani medium) with kanamycin (50 µg/mL) were washed three times in plain LB, and resuspended in a volume of LB representing approximately a 60-fold concentration of the original culture volume. Recipient cells, a rifampicin resistant subculture of *Methylomonas sp. 16a* (designated 16aA), were grown for 48 hours in BTZ-3 (Table 1) with rifampicin (50 µg/mL) under 25% methane, washed three times in BTZ-3, and resuspended in a volume of BTZ-3 representing approximately a 150-fold concentration of the original culture volume. The resulting donor, helper, and recipient cell pastes were combined on the surface of BTZ-3 agar plates containing 0.5% (w/v) yeast extract in ratios of 1:1:2 respectively. Plates were maintained at 25% methane for 16-24 hours to allow conjugation to occur. Cell pastes were harvested and resuspended in BTZ-3. Serial dilutions were plated on BTZ-3 agar with rifampicin (50 µg/mL) and kanamycin (50 µg/mL). Resulting colonies were patched to new selective plates. Colonies which grew the second time were then transferred to liquid BTZ-3 with rifampicin (50 µg/mL) and kanamycin (50 µg/mL). The presence of the limonene synthase gene in the 16a conjugates was verified by PCR using primer set 5'-atgagacgatccggaactacaaccc-3' (SEQID NO:1) and 5'-tcattgcaaagggtcgaataagggttctgg-3' (SEQID NO:2) which anneals to the N- and C-terminus of the limonene synthase respectively. The primer set 5'-atgattgaacaagatggattgc-3' (SEQID NO:3) and 5'-aagctttcaaaagaactcgtc-3' (SEQID NO:4) was used to detect kanamycin resistance gene as a control.

EXAMPLE 7

Limonene Detection.

Liquid cultures of *Methylomonas sp. 16a* transconjugants were grown in airtight bottles for approximately 48 hours in BTZ-3 supplemented with rifampicin (50 µg/mL), kanamycin (50 µg/mL) and 25% methane. Contents of the culture vessel were harvested for limonene and analyzed by gas chromatography (GC) analysis. Briefly, ethyl acetate was injected through the septa in order to extract limonene from both the headspace

and from the liquid. Samples were agitated for 5 min., transferred to appropriate centrifuge tubes and centrifuged to achieve complete separation of the organic and aqueous phases. The organic phase was removed and loaded onto a DB-1 column (30M/0.25 ID/0.25 film thickness, J&W Scientific, Folsom, CA).

The initial oven temperature was set at 50°C and temperature was increased to 250°C at a rate of 10 degrees per minute. After 5 minutes at 250°C, the samples were injected into the column. A Septum purge was applied for one minute at a rate of 2.4 mL per minute. The runs were carried out at a head pressure of 8 psi and flow rate of 24.5 mL per minute. Limonene used as a standard was purchased from Sigma-Aldrich (Cat#18,316-4). Samples analyzed by GC/MS are identical to those analyzed by GC alone. GC/MS instrument type: ProSpec. Source type: Combined-EI/CI SRC. Electron energy: 69.0 eV. Multiplier voltages: #1: 198.6 V, #2: 250.0 V, #3: 250.0 V, #4: 250.0 V. Emission current: 0.0 mA Trap current: 444.1 µA. Ion repeller: 5.7 V. Source temp: 225.3°C. Extraction heater current: 0.0 mA. Slit criteria: Source: 47.8%, Collector: 50.4%, Alpha: 100%, Z2 Restrict: 0.0%, Z3 Restrict: 0.0%, Z4 Restrict: 0.0%. Lens Criteria: Ion Energy: 1.4, Focus#1: 2.2 V, Beam Centre: -48.7 V, Focus #2: 5.8 V, Y-Focus: 4.4 V, Y-Def#1: -88.0 V, Z-Def#1: 7.5 V, Rotate #1: 0.0 V, Z-Def#2: -12.6 V, Z-Focus #2: 6.0V, Rotate #2: -13.6 V, Curve #2: -4.0V, Curve #3: -8.6 V, Rotate #3: -9.0 V, Z-Focus #3: 9.3 V, Z-Def #3: 0.0 V, Rotate #4: 0.0 V. Magnet parameters: IMR 1160.16, Standard coil. No ramped parameters. HP6890 GC parameters: Automatic restart, Capillary line temp (1): 240.0°C, Capillary line temp (2): 240.0°C, Reentrant temp: 240.0°C, Maximum oven temp: 325.2°C, Equilibrium time: 0.2 min, Oven Temperature Ramps: Temp #1: 50.0°C, Time #1: 1.0 min, Rate #1: 10.0°C/min, Temp#2: 200.0°C, Time #2: 1.0 min, Rate #2: 10.0°C/min, Temp #3: 250.0°C, Time #3: 10.0 min, Rate #3: 0.0°C/min. Injectors: "A": Active, Injector "A" temp: 270.0°C, Injection type: splitless, Purge "A" on time: 1.0 min, Purge "A" Flow: 1.0 mL/min, Col 1 Const Flow: 1.0 mL/min. "B": Active, Injection type: Cool-on-Col, "B" Injector Temp Ramps: Temp #1: 50.0°C, Time #1: 650.0 min, Rate #1: 0.0°C/min, Col 1 Const flow: 2.0mL/min.

Methylomonas sp. 16a which received the limonene synthase gene produced limonene (~0.5 ppm). In the GC analysis, chromatograms of these extracts display a peak which is superimposable with that of the

